

Original Article

Hemopoietic effect of extracts from constituent herbal medicines of Samul-tang on phenylhydrazine-induced hemolytic anemia in rats

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Abstract: Samul-tang (Si-Wu-Tang, SMT), a kind of herbal medicines, has been used for the hemato-deficient disease for hundreds of years. In this work, investigate the anti-anemia activity of the H₂O extracts from constituent herbal medicines of Samul-tang in an anemia model induced by intravenous injection of phenylhydrazine-HCL (PHZ) at 10 mg/kg for 4 days. After PHZ injection, female Sprague-Dawley rats were administered extracts from constituent herbal medicines of SMT (300 mg/kg/day, p.o.) daily for 1 week. Results showed that severe hemolysis was induced by PHZ. For *Paeonia lactiflora* (PL2) H₂O extract treated groups, the concentration of hemoglobin, hematocrit and red blood cells number increased much more significantly than PHZ-treated group. Moreover, *Angelica gigas* (AG), *Angelica acutiloba* (AA), *Paeonia lactiflora* (PL2) and *Rehmannia glutinosa* (RG) extract administration significantly improved serum erythropoietin concentration. The activity of aminolevulinic acid dehydratase (ALDL) in liver homogenate was increased in *Angelica gigas*(AA), *Paeonia lactiflora* (PL2) and *Rehmannia glutinosa* (RG) treated group.

Keywords: Samul-tang (Si-Wu-Tang, SMT), herbal medicine, phenylhydrazine-HCL (PHZ), anemia, hemoglobin, hematocrit

Introduction

Anemia causes hypoxia due to a failure to meet tissue oxygen demand. It is a disease due to a decrease in hemoglobin (Hgb) inside erythrocytes (RBCs) or a deficiency of RBCs, which provide oxygen to peripheral tissues. Anemia can be separated into morphological and causal categories [1]. The morphological category depends on the size of hemocytes and Hgb concentration, whereas the causal category consists of hypochromic anemia and hemolytic anemia due to hemorrhagic anemia and aplastic anemia and nutritional deficiency of RBCs (iron, vitamin B12, and folic acid) [2]. Hemorrhagic and hypochromic anemia are cured by supplementing the blood and by nutritional means and are eliminated by investigating the causes for the hemolytic anemia. No special treatments for essential aplastic ane-

mia and other anemias are available currently, and these diseases are treated by restoring bone marrow using crude drugs. Anemia animal models include a low-iron diet, and the other model is induced by vitamin B12 and folic acid deficiency. However, these models require a long induction time; thus, anemia is generally induced by cyclophosphamide, an anti-cancer agent, or by phenylhydrazine (PHZ). PHZ is a strong oxidant and its metabolites include reactive oxygen species, phenyldiazene, the phenylhydrazyl radical, and benzenediazonium ions through automatic oxidation, which damages RBCs by oxidation and causes severe hemolytic anemia by oxidating HbG [3-5]. Oriental medicine has utilized Samul-tang as a representative treatment for blood-related diseases including anemia. The treatment was first reported in "Taepyeonghyeminhwajegukbang", and has been applied to supplement and rein-

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Table 1. Basic information for raw herbal materials used in this study

Herbal name	Scientific name	Place of product	Abbreviation name
Angelicae Gigantis Radix	<i>Angelica gigas</i>	Korea	AG
	<i>Angelica sinensis</i>	China	AS
	<i>Angelica acutiloba</i>	Korea	AA
Cnidii Rhizoma	<i>Ligusticum chuanxiong</i>	Korea	CRL
	<i>Cnidium officinale</i>	Korea	CRC
Paeoniae Radix	<i>Paeonia lactiflora</i>	China	PL1
	<i>Paeonia lactiflora</i>	Korea	PL2
Rehmanniae Radix Preparata	<i>Rehmannia glutinosa</i>	Korea	RG

vigorate the bloodstream. Samul-tang consists of Angelicae gigantis radix, Cnidii rhizoma, Paeoniae radix and Rehmanniae radix preparata. *A. gigantis* Radix is a perennial plant in the Umbelliferae family, and its dried roots are used. The originating plants are *Angelica gigas* Nakai in Korea, *Angelica sinensis* (Oliv.) Diels in China, and *Angelica acutiloba* Kitagawa in Japan [6-9]. *L. wallichii* Franch is a perennial plant in the Umbelliferae family. Its origins in China and Japan are *Ligusticum chuanxiong* Hort and *Cnidium officinale* Makino, respectively. *P. lactiflora* Pallas is a perennial plant in the Paeoniaceae family, and its dried roots are used. Its origin is China, and there are a number of allied species with the same genus but *P. lactiflora* Pallas is mainly grown. The boiled roots of white *P. lactiflora* Pallas are dried and used after eliminating the shell and red *P. lactiflora* Pallas is dried with the shell. *Rehmannia glutinosa* var. *hueichingensis* is processed from roots of *R. glutinosa* Liboschitz and is a plant in the Scrophulariaceae family that originated in China. Existing studies on four substances decocted from these plants indicate that they improve anemia and have anti-cancer capability [10-14]. However, decoction is rarely used as a basic treatment in clinical cases. Rather, it is variously used depending on symptoms, and no studies on the decoctions from these plants have been conducted for the purpose of medical research on anemia. In this study selected four decoctions from these plants and tested their effects on anemia in rats.

Materials and methods

Sample extraction and preparation

The plants for decoction included *A. giantis* radix, *L. wallichii* Franch, *P. lactiflora* Pallas,

and *R. glutinosa* var. *hueichingensis*. Eight species, including three species of *A. giantis* radix (*Angelica gigas* Nakai, *Angelica sinensis* (Oliv.) Diels, *Angelica acutiloba* Kitagawa), two species of *L. wallichii* Franch (*Cnidium officinale* Makino and *Ligusticum chuanxiong* Hort), two *P. lactiflora* Pallas (white Paeoniaceae and red *P. lactiflora* Pallas), and one *R. glutinosa* var. *hueichingensis* were purchased from local and foreign markets. Then, the substances were tested after being extracted at Wooseok University (Table 1). The plants were powdered and 150 g of each was reflux circulated twice at 2 hour intervals in 1.5 L of deionized water, which was about 10 times more than the sample. After the extraction, the filtered extracts were frozen, powdered, and stored at -70°C. The powdered extracts were rehydrated and diluted immediately before the experiment.

Experimented animals

Sprague-Dawley rats (4-week old, females) were purchased from CoreTec (Gyeonggi, Korea) and acclimated for 1 week under 12 h light: 12 h dark conditions in a room with constant temperature (20 ± 2°C) and humidity (50 ± 5%). Feed and drinking water were provided freely. Healthy animals after the 1 week adaptation were used for the experiment, and all procedures were performed after obtaining approval from the animal experiment ethics committee at the Korea Institute of Oriental Medicine.

Inducing anemia and injections

Anemia was induced by venoclysis in rat tails for 4 days at a concentration of 10 mg/kg using melted phenylhydrazine (PHZ: Sigma Chemical Co., St. Louis MO, USA) in sterilized normal saline. The rats were divided into 10 groups

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Table 2. Weight gain, food intake and food efficiency ratio of experimental animal

	Control	PHZ	AG	AS	AA	CRL	CRC	PL1	PL2	RG
Final weight (g)	134.76 ± 12.70	129.62 ± 3.84#	133.9 ± 9.77	135.78 ± 7.19	135.18 ± 12.48	138.62 ± 6.41	135.02 ± 9.95	138.88 ± 5.99	145.14 ± 5.06	132.36 ± 5.56
Weight gain (g/day)	2.79 ± 0.42	2.46 ± 0.25	3.91 ± 0.82	3.90 ± 0.36	2.88 ± 0.64	3.85 ± 0.64	4.10 ± 0.56	4.64 ± 0.44	5.68 ± 0.67	2.89 ± 0.54
Food intake (g/day)	18.94 ± 0.68	18.27 ± 0.94	19.16 ± 1.02	19.78 ± 0.88	19.42 ± 0.96	19.06 ± 0.44	18.38 ± 0.64	18.82 ± 0.51	18.48 ± 1.27	18.82 ± 0.99
FER (food efficiency ratio)	0.15 ± 0.05	0.13 ± 0.04	0.20 ± 0.06	0.20 ± 0.06	0.15 ± 0.03	0.20 ± 0.07	0.22 ± 0.06	0.25 ± 0.03	0.31 ± 0.03*	0.15 ± 0.04

*P < 0.05, significantly different from negative control. Results are expressed as mean ± SD (n = 5).

Table 3. Effect of Samul-tang extracts on hematological indices in PHZ-induced hemolytic anemia in rat

	Control	PHZ	AG	AS	AA	CRL	CRC	PL1	PL2	RG
RBC (×10 ⁶ mm ³)	6.49 ± 0.24	5.69 ± 0.26	5.91 ± 0.46	5.69 ± 0.17	5.90 ± 0.11	5.87 ± 0.27	5.77 ± 0.15	5.85 ± 0.29	6.25 ± 0.22*	5.91 ± 0.06
WBC (×10 ³ mm ³)	8.48 ± 1.68	7.91 ± 2.20	7.13 ± 0.88	9.21 ± 2.40	8.58 ± 2.61	8.77 ± 0.92	7.83 ± 1.12	7.54 ± 1.00	8.23 ± 0.64	7.44 ± 1.61
MCH (pg)	20.96 ± 0.21	22.6 ± 0.53	22.70 ± 0.62	22.84 ± 0.83	22.24 ± 0.48	22.78 ± 1.17	22.58 ± 0.68	22.5 ± 0.86	22.5 ± 0.18	22.88 ± 0.43
MCV (fL)	68.16 ± 1.40	75.36 ± 1.94	76.02 ± 1.65	76.44 ± 3.61	73.46 ± 1.59	74.5 ± 1.70	75.04 ± 1.61	75.62 ± 2.56	75.8 ± 2.22	75.66 ± 1.76
MCHC (g/dL)	30.74 ± 0.34	29.98 ± 0.29	29.82 ± 0.33	29.92 ± 0.43	30.28 ± 0.49	30.56 ± 1.09	30.1 ± 0.41	29.76 ± 0.39	29.73 ± 0.98	30.26 ± 0.44

RBC: red blood cell, WBC: white blood cell, MCH: mean corpuscular hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration. Results are expressed as mean ± SD (n = 5).

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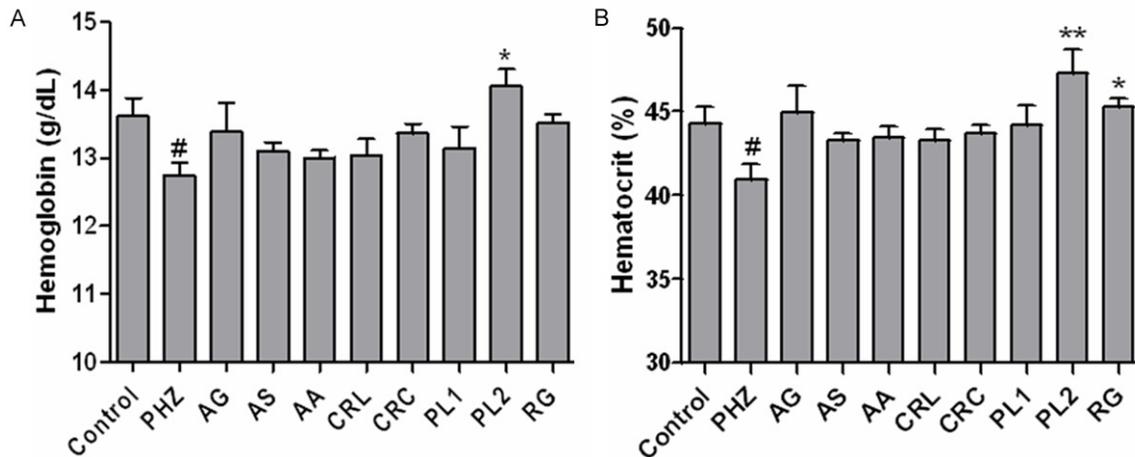


Figure 1. Effect of Samul-tang extracts on hemoglobin and hematocrit in whole blood PHZ-induced hemolytic anemia rats. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, #significantly different from normal control, *significantly different from negative control. Results are expressed as mean \pm SD ($n = 5$).

including a normal control group, control anemia group treated with PHZ and other groups injected with 300 mg/kg of the extracts after the PHZ treatment. The extracts were administered orally administration for 1 week after anemia was induced in normal saline, with the same amount of saline administered orally to the normal control group and the PHZ-treated anemia control group.

Diet efficacy

Diet intake during the experiment was measured twice at the start and ending dates of administration, and weights were measured three times every 3 days during the experiment. The food efficiency ratio was calculated by dividing the weight gained by diet intake for the total experimental period.

Blood analyses

Rats were anesthetized with zoletil (Virbac S.A, Paris, France) and their blood was taken from the abdominal aorta after anesthesia. Blood was collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes NJ, USA) coated with EDTA anti-coagulant. RBCs, Hbg, Hct, mean corpuscular volume, (MCV) and mean corpuscular hemoglobin (MCH) concentrations were measured with an automatic blood cell counter (XE 2100D, Sysmex, Kobe, Japan).

Erythropoietin (EPO)

Serum EPO concentrations were measured in accordance with the recommendation from the

manufacturer of the Rat EPO Enzyme-linked Immunosorbent Assay (ELISA) kit (Wuhan EIAab Science Co., Guangguguoji, Wuhan, China), and the results were compiled by absorbance at 450 nm using a microplate reader (Molecular Device Co., Sunnyvale, CA, USA).

Activating δ -aminolevulinic acid dehydrate (ALAD)

The ALAD concentration in the liver tissue was measured based on the manufacturer's recommendations from the Rat Aminolevulinic Acid Dehydratase EPO ELISA kit (Wuhan EIAab Science Co.), and the results were measured by absorbance at 450 nm using a microplate reader.

Statistical analysis

Results are presented as the average \pm standard deviation, and the differences among test groups were assessed by one-way analysis of variance followed by Tukey's multiple comparison test using Graphpad prism 5.0 software (Graph pad, La Jolla, CA, USA). A $P < 0.05$ was considered significant.

Results

Diet efficacy of the animals

After the experiment, the anemia-induced group injected with PHZ showed a significant decrease in weight compared to that in the normal group ($P < 0.05$). The groups treated with plant extracts showed a similar increase as

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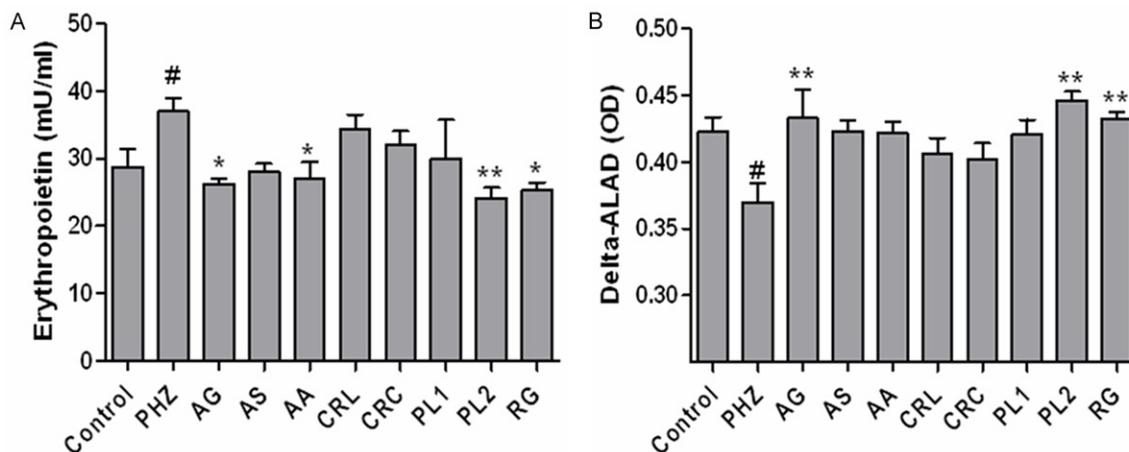


Figure 2. Effect of Samultang extracts on erythropoietin and δ -ALAD in PHZ-induced hemolytic anemia rats. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, #significantly different from normal control, *significantly different from negative control. Results are expressed as mean \pm SD ($n = 5$).

that observed in the normal control group, and the group administered the PL2 extract showed the greatest weight increase (**Table 2**). None of the groups showed different dietary intake but the efficacy of PL2 extract group was significantly higher ($P < 0.05$). Weight loss in the PHZ-treated group suggested that nutrition deteriorated due to oxidation losses in tissues from oxygen free radicals due to the automatic oxidation of PHZ, a strong oxidant [15].

Hbg and Hct levels

Hbg and Hct levels were measured in whole blood (**Figure 1**). Hbg concentration decreased to 90.32% in the PHZ group which showed hemolytic anemia due to the 4 days of PHZ injections compared to that in the normal control group, suggesting that the anemia was induced ($P < 0.05$). The results of groups treated with the extracts after inducing hemolytic anemia showed a significant increase in the PL2-treated group ($P < 0.05$) but no significant difference was found in the AG treatment group injected with *A. gigantis* radix extract or the CRC group injected with the *L. wallichii* Franch extract despite a slight increase. The Hct level decreased significantly in the PHZ treatment group compared with that in the normal control group ($P < 0.05$). All groups treated with the plant extracts tended to show increased Hct levels compared to that in the PHZ treatment group, but the difference was not significant. However, the PL2 treatment ($P < 0.01$) and the RG treatment ($P < 0.05$) groups showed signifi-

cantly higher Hct levels. Hbg content and Hct level in the PL2 and the RG treated groups were restored to normal levels, indicating that the extracts were effective for treating the anemia. In addition, the AG treatment group aided in the treatment of hemolytic anemia, but the result was not significant.

Hematological analysis

The number of RBCs in the PHZ control group decreased by about 17% compared to that in the normal control group ($P < 0.001$, **Table 3**). As shown in **Figure 1**, the decreased number of RBCs caused a decrease in Hbg and Hct levels. All groups except the AA treatment group showed increased RBCs compared to those in the PHZ treatment group. In particular, the PL2 group showed a significant increase compared to that in the PHZ treatment group and the AG and the AA groups of *A. gigantis* Radix and the CRC treatment group of *L. wallichii* Franch were effective for increasing RBCs. The RG treatment group showed no significant changes but increased more than that in the PHZ treatment group. The number of RBCs did not increase or decrease in all groups, nor did MCH, MCV, and MCH concentrations.

EPO content and ALAD activation

Figure 2 shows the effects of the plant extracts serum EPO content. Serum EPO content in the PHZ treatment group, which had hemolytic anemia following the PHZ injection, increased by

15% compared to that in the normal treatment group. The inhibition of the increase in serum EPO by the extracts decreased the PHZ-induced hemolytic anemia. Serum EPO content increased in the normal control group followed by the RG, AG and AA extracts, indicating that decreasing oxygen tension in the aorta from the decreased number of RBCs and Hbg due to anemia increased EPO in the kidney and stimulated RBC production from bone marrow [15, 16]. In particular, the PL2 extract resulted in increased number of RBCs and Hbg content to the normal level (Table 3). ALAD is an enzyme that catalyzes the synthesis the porphobilinogen from δ -aminolevulinic acid (ALA), and is the initial synthesis process for Hbg [17]. Hemolytic anemia inhibits ALAD activation and increases urinary excretion of ALA to inhibit hematopoiesis. The effectiveness of the extracts against PHZ-induced hemolytic anemia was measured (Figure 2). ALAD activation decreased by 20% in the PHZ treatment group compared to that in the normal control group but ALAD activation increased significantly in the PL2 group compared to that in the PHZ treated group ($P < 0.01$).

Discussion

The purpose of the study was to verify the effects of plant extracts on hematopoiesis in rats following hemolytic anemia induced by PHZ injection. A 10 mg/kg dose of PHZ was injected for 4 days through venoclysis in 4 weeks old female Sprague-Dawley rats to induce hemolytic anemia and 300 mg/kg of eight different hot water extracts were orally administered. The PHZ group showed hemolytic anemia and a significant decrease in Hbg and Hct compared to that in the normal control group. Groups treated with the plants extract showed increased Hbg, and Hct levels and RBCs. In particular, *P. lactiflora* Pallas significantly increased (*P. lactiflora* Pallas and *R. glutinosa* var. *hueichingensis*) and *A. gigas* Nakai in *A. gigantis* Radix showed an insignificant increase. The increased serum EPO content following the PHZ injection was restored to the normal level in all the groups except the *L. wallichii* Franch extract treatment group. ALAD synthesis increased in the group treated with the white *P. lactiflora* Pallas extract. Therefore, some of these plants extracts are functional materials with desirable hematopoiesis properties for hemolytic anemia induced by PHZ. In particular, the oriental combination of *A. gigas*

Nakai and the white *P. lactiflora* Pallas stimulated the blood parameter most effectively.

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Disclosure of conflict of interest

None.

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